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14. ABSTRACT

To build a more robust microbe for production of D-1,2,4-butanetriol with minimal amount of byproducts formation, various experimental parameters including microbial genetic make-up, media component and fermentation reaction condition were investigated. The investigation provided an optimized E. coli biocatalyst KIT18/pWN7.126B carrying a codon-optimized Pseudomonas putida mdlC benzoylformate decarboxylase and an alcohol dehydrogenase, adhP capable of producing an upward of 35 g/L of D-1,2,4-butanetriol from D-xylose. However the process also generates byproducts, such as, 3,4-dihydroxy-D-butanol and 3,4-dihydroxy-D-butanoic acid, that are difficult to separate and purified. To circumvent this problem and eliminate the need of using xylose as a carbon source, a new two-step approach to D-1,2,4-butanetriol synthesis from glucose has been developed. The process relies on the use of biocatalyst E. coli, WY9/pWY1, for first converting D-glucose to D-xylonic acid, which is then utilized by E. coli biocatalyst DH5x/pWN6.186A, which carries a codon optimized P. putida mdlC plasmid encoding benzoylformate decarboxylase while relying on native D-xylonate transport along with native D-xylonate dehydratase and dehydrogenase activities for the synthesis of D-1,2,4-butanetriol. The two-step process has been demonstrated as capable of producing D-1,2,4-butanetriol from glucose, a much more reliable source of feedstock, successfully.

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Abstract/Project Summary: To build a more robust microbe for production of D-1,2,4-butanetriol with minimal amount of byproducts formation, various experimental parameters including microbial genetic make-up, media component and fermentation reaction condition were investigated. The investigation provided an optimized *E. coli* biocatalyst KIT18/pWN7.126B carrying a codon-optimized *Pseudomonas putida mdlC* benzoylformate decarboxylase and an alcohol dehydrogenase, *adhP* capable of producing an upward of 35 g/L of D-1,2,4-butanetriol from D-xylose. However the process also generates byproducts, such as, 3,4-dihydroxy-D-butanol and 3,4-dihydroxy-D-butanoic acid, that are difficult to separate and purified. To circumvent this problem and eliminate the need of using xylose as a carbon source, a new two-step approach to D-1,2,4-butanetriol synthesis from glucose has been developed. The process relies on the use of biocatalyst *E. coli*, WY9/pWY1, for first converting D-glucose to D-xylonic acid, which is then utilized by *E. coli* biocatalyst DH5x/pWN6.186A, which carries a codon optimized *P. putida mdlC* plasmid encoding benzoylformate decarboxylase while relying on native D-xylonate transport along with native D-xylonate dehydratase and dehydrogenase activities for the synthesis of D-1,2,4-butanetriol. The two-step process has been demonstrated as capable of producing D-1,2,4-butanetriol from glucose, a much more reliable source of feedstock, successfully.

Scientific Technical Objectives: One of the major byproduct formed during microbial D-1,2,4-butanetriol synthesis is 3,4-dihydroxy-D-butanoic acid. Successful elimination of this byproduct formation is not only crucial to facilitate downstream purification, but also to potentially increase our D-1,2,4-butanetriol production. The current microbial synthesis of D-1,2,4-butanetriol using *E. coli* KIT18/pWN7.126B is based on the use of D-xylose as the starting material. Although D-xylose is abundant in hemicellulose, streams of D-xylose sufficiently pure to support microbial growth are not available in the U.S. Because of the current expense of D-xylose, a microbial synthesis of D-1,2,4-butanetriol from D-glucose was targeted for development. The activity of *mdlC*-encoded benzoylformate decarboxylase is essential to improving the yields and concentrations of microbe-synthesized D-1,2,4-butanetriol. Codon optimization of *Pseudomonas putida mdlC* for heterologous expression in an *E. coli* host was therefore pursued. Reaction engineering to improve microbial synthesis of D-1,2,4-butanetriol titer and yield from D-xylose was also examined.

Approach: One of the strategies to understand this byproduct formation requires identification of the enzyme and encoding gene responsible for the conversion of 3,4-dihydroxy-D-butanal into D-1,2,4-butanetriol. Overexpression of the identified dehydrogenase was anticipated to reduce or eliminate formation of byproduct 3,4-dihydroxy-D-butanoic acid. Screening of NAD- and NADP- dependent 1,2,4-butanetriol dehydrogenases in *E. coli* was therefore pursued. Employing reaction engineering in the current fermentor-controlled microbial synthesis of D-1,2,4-butanetriol from D-xylose, *E. coli* KIT18/pWN7.126B was evaluated under different conditions. First step towards assembling an *E. coli* construct capable of synthesizing D-1,2,4-butanetriol from D-glucose is to create a pathway for the conversion of D-glucose into D-xylonic acid. The isozymes of D-ribose 5-phosphate isomerase encoded by *rpiA* and *rpiB* and the isozymes of transketolase encoded by *tktA* and *tktB* in *E. coli* were the focus of research activities. Inactivations of various combinations of these loci were examined in constructs where *yfbT*-encoded sugar phosphatase activity was overexpressed. *E. coli* WY9/pWY1 was created that synthesized 2 g/L of D-xylonic acid in rich LB-xylose medium. Defined minimal salt medium was formulated to enable high-density cultivation of WY9/pWY1 under fed-batch fermentor-controlled conditions. To increase the activity of *mdlC*-encoded benzoylformate decarboxylase, the codons for this *P. putida* gene were optimized *in silico* for the expression in *E. coli*. Codon-optimized *mdlC* was chemically synthesized and the impact of codon optimization on the specific activity of heterologously expressed benzoylformate decarboxylase was examined in *E. coli* cultured under fermentor-controlled

conditions. In parallel with these efforts, microbial synthesis of D-1,2,4-butanetriol from D-glucose using a single *E. coli* microbe was also examined.

Accomplishments: *E. coli adhP, adhE* and *yiaY* encoding substrate-nonspecific alcohol dehydrogenases were found active towards using 3,4-dihydroxy-D-butanal as substrate. Among these dehydrogenases, the importance of native *E. coli* AdhP in the D-1,2,4-butanetriol biosynthesis was demonstrated using the chromosomal *adhP* inactivation strategy. *E. coli adhP* knockout KIT10/pWN7.126B synthesized only 6.5 g/L D-1,2,4-butanetriol, which accounted to a 40% decrease in D-1,2,4-butanetriol production when comparing to its parent microbe WN13/pWN7.126B. A second generation of D-1,2,4-butanetriol biocatalyst KIT18/pWN7.126B was created by overexpressing *adhP* chromosomally. Coupling reaction engineering and the use of this new biocatalyst led to a substantial improvement in D-1,2,4-butanetriol titer. *E. coli* KIT18/pWN7.126B synthesized 35 g/L at 50% mol/mol yield of D-1,2,4-butanetriol from D-xylose under fed-batch fermentor-controlled conditions. Improved D-1,2,4-butanetriol production and reduced organic acid byproducts formation were observed. Triple knockouts *E. coli* WY9 (*tktA rpiA rpiB*)/pWY1 synthesized 5.5 g/L of D-xylonic acid from D-glucose using defined minimal medium under fed-batch fermentor-controlled conditions when supplemented with D-ribose. A two-step microbial synthesis of D-1,2,4-butanetriol from D-glucose was formulated using *E. coli* WY9/pWY1 and DH5/pWN6.186A as biocatalysts. Attempt to synthesize D-1,2,4-butanetriol from D-glucose in a single step using *E. coli* WY9/pWN7.126B was unsuccessful. Codon-optimized *P. putida mdlC* expressed in an *E. coli* host used for synthesis of D-1,2,4-butanetriol resulted in higher specific activities of benzoylformate decarboxylase relative to heterologous expression of wild-type *mdlC* in the same *E. coli* host when these constructs were cultured under fermentor-controlled conditions.

Conclusions: It is possible to use glucose as the input carbon source for the production of 1,2,4-butanetriol, and to avoid the use of the much more expensive xylose as starting material. While significant improvement in the overall process for the production of butanetriol was realized, it is clear that further work is required to adjust the redox balance within the micro-organism in order to completely prevent production of 3,4-dihydroxypropionic acid, and maximize the production of butanetriol. This might be accomplished by the adjustment of carbon flux from glycolysis into the pentose phosphate pathway via introduction of the enzyme glucose-6-phosphate dehydrogenase known as the zwischenferment enzyme or zwf.

Significance: 1,2,4-Butanetriol remains of commercial interest, and Draths has received requests for supplying this material. Draths now has demonstrated two processes capable of production of 1,2,4-butanetriol. One, the single-step fermentation using KIT18/pWN7.126B with improved production of 1,2,4-butanetriol form xylose will become increasingly valuable as the biorefinery industry continues to make progress in the isolation of xylose from hemi-cellulose. The other, two-step process using WY9/pWY1 and DH5a/pWN6.186A is capable of 1,2,4-butanetriol production from glucose. This allows Draths to utilize all sugars streams from biomass pre-treatment and hydrolysis technologies.

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